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## **Chemical targeting of NEET proteins reveals their function in mitochondrial morphodynamics.**

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## **Abstract**

Several human pathologies including neurological, cardiac, infectious, cancerous and metabolic diseases have been associated with altered mitochondria morphodynamics. Here we identify a small organic molecule, we named Mito-C, which is addressed to mitochondria and rapidly provokes mitochondrial network fragmentation. Biochemical analyses reveal that Mito-C is a member of a novel class of heterocyclic compounds that target the NEET protein family, previously reported to regulate mitochondrial iron and ROS homeostasis. One of the NEET proteins, NAF-1, is shown for the first time to be an important regulator of mitochondria morphodynamics by facilitating recruitment of DRP1 to the ER-mitochondria interface. Consistently with observation that certain viruses modulate mitochondrial morphogenesis as a necessary part of their replication cycle, Mito-C counteracts Dengue induced mitochondrial network hyperfusion and represses viral replication. The novel chemical class to which Mito-C belongs is of therapeutic relevance in pathologies where altered mitochondria dynamics is part of disease etiology and NAF-1 is highlighted as an important therapeutic target in antiviral research.

## Introduction

Mitochondria are double membrane organelles essential for energy homeostasis in eukaryotes but also critical for regulating iron and calcium homeostasis, redox regulation, autophagy, innate immunity and cell death [1]. A broad range of diseases, including viral infections, have been associated with irregular mitochondria morphology and dynamics, highlighting the central role of mitochondrial network dynamics in the maintenance of cellular homeostasis [2]. Regulation of mitochondrial morphology is a consequence of a balance between fission and fusion events that maintain mitochondria number, size and shape [3]. This dynamic equilibrium enables adaptation to a wide range of stress situations. Moreover, mitochondria are physically bound to the endoplasmic reticulum (ER), via ER-Mitochondrial contact sites (ER-MTcs). ER-MTcs engage multiple proteins, from both ER and mitochondria and are involved in a wide range of cellular functions, including autophagosome biogenesis and mitochondrial fission. A critical regulator of the mitochondrial fission process is the cytosolic GTPase dynamin-related protein 1 (DRP1), which relocates to the mitochondrial surface to promote mitochondrial network fragmentation [4]. Interestingly, DRP1 recruitment to ER-MTcs defines the position of the mitochondrial division site [5], although how this directed recruitment occurs still remains unclear.

In addition to proteins involved in fission/fusion regulation, many other proteins of critical importance to cell function such as the respiratory chain, innate immunity, redox regulation and iron homeostasis are located on or in the mitochondria. Among these mitochondria-associated proteins are members of the NEET family which regulate iron and reactive oxygen species (ROS) homeostasis in the mitochondria [6–8]. There are three NEET proteins: MitoNEET, NAF-1 and MiNT and they are reported to localize at the mitochondria matrix (MiNT), the outer-mitochondrial membrane (MitoNEET and NAF-1) and the Endoplasmic Reticulum (NAF-1). Their unique ability to reversibly bind the redox active [2Fe-2S] cluster enables their function as iron-sulfur transfer proteins transferring [2Fe-2S] clusters out of the mitochondria [9,10]. MitoNEET has been associated with the regulation of oxidative phosphorylation in mitochondria [11] and deletion of its gene (*cisd1*) alters the integrity of inter-mitochondrial junctions [12]. In human, frame shift mutations in the *cisd2* gene encoding NAF-1 result in the autosomal recessive disorder Wolfram Syndrome Type 2 (WFS2), characterized at the cellular level by mitochondria dysfunctions, iron accumulation in mitochondria, increased autophagy and cell death. In contrast to MitoNEET and NAF-1, MiNT is less characterized. Recently the crystal structure of MiNT has been resolved and its contribution to tumorigenesis explored, especially in correlation with iron and ROS

homeostasis in cancer cells [13]. Despite the numerous studies connecting NEET proteins to different mitochondrial disorders, no functional connection has been made to infectious diseases.

Here we find that Mito-C, a compound not previously described, targets the NEET protein NAF-1, modifies mitochondrial morphology and dynamics and is able to counteract Dengue-induced mitochondria network hyperfusion and to strongly reduce Dengue virus replication. Furthermore, via pharmacological and genetic approaches we here also reveal the central role of NEET proteins in regulation of mitochondrial membrane dynamics via the ER-mitochondria associated fission machinery.

## Results

We synthesized a novel small molecule, Mito-C (2-[(3,4-Dimethoxybenzoyl)amino]-6,6-dimethyl-5,7-dihydro-4H-benzothiophene-3-carboxylic acid, Fig1a) that was selected for its ability to affect the morphology of the mitochondrial network without adverse impact upon cell viability. The mitochondrial morphology modification was quantified using an unbiased image analysis method based on an immunofluorescence parameter termed skewness. This reflects the symmetry of the network as determined by the distribution of mitochondrial fluorescent staining in 2D space (FigS1a). In a dose dependent manner (0.2 $\mu$ M to 2 $\mu$ M, FigS1b and S1c), Mito-C rapidly induces mitochondrial network fragmentation (Fig1b and 1c), with no evidence of cell death following much longer treatment (24 hours) at concentrations up to 20 $\mu$ M (FigS2a and S2b). The Mito-C associated mitochondrial fragmentation observed after 15 minutes of treatment was reversible, with cells recovering normal mitochondria following wash out and overnight culture (FigS2c and S2d).

To determine the protein binding target of Mito-C we used a photo-affinity labeling based method (Capture Compound Mass Spectrometry) to identify the cellular target of Mito-C. Mito-C was synthesized with a photo-reactive function for covalent cross-linking to bound protein and a sorting function for isolation of bound protein and identification by mass spectrometric analysis [14]. This approach revealed interaction of the capture compound with the NEET protein MiNT. To examine the functional effect of Mito-C upon NEET proteins, possible effect upon the rate of release of the [2Fe-2S] cluster from each purified NEET protein was tested with or without Mito-C or an inactive analogue Mito-N (a structural analogue of Mito-C with no effect upon the morphology of the mitochondrial network). As shown in Fig1d (for NAF-1) and FigS3 (for MitoNEET and MiNT) the presence of Mito-C significantly enhanced the stability of NAF1, MitoNEET and MiNT for their bound [2Fe-2S] cluster thus in effect inhibiting their Fe/2Fe-2S transfer function. Such a functional test proved the physical binding of Mito-C to NEET proteins.

MiNT and MitoNEET have both been reported to localize exclusively to the mitochondrial matrix and outer membrane respectively [15]. However, NAF1 has been reported to localize to both the ER ([16] [15] and FigS4a), to the mitochondria-associated membranes [17] and MT outer membranes [18] and FigS4b). We now show that NAF-1 is localized at ER and ER-MT interface subdomains (FigS4c) using PTPIP51 as marker of ER-MTcs [19,20]. To strengthen the hypothesis that Mito-C elicits its effects in cells by modulating NEET protein function, a fluorescent-tagged version of Mito-C (<sup>fluo</sup>Mito-C) was generated by linking the 7-nitrobenzofurazan green fluorescent tag on the para position of the right hand phenol ring (Fig 1a). Live microscopy imaging revealed that <sup>fluo</sup>Mito-C targets both mitochondria (MT) and

Endoplasmic Reticulum (ER) as illustrated by the colocalization between <sup>fluo</sup>Mito-C and the MT live probe mitotracker RedOx or the ER marker Sec61-RFP (FigS5a and FigS5b). The colocalization observed between the NAF1-RFP and <sup>fluo</sup>Mito-C further indicates that the NEET proteins and Mito-C are localized to the same cellular compartments (Fig1e). Altogether, our data indicate that Mito-C targets NEET proteins by inhibiting their [2Fe-2S] transfer function and initiates immediate and reversible MT network fragmentation.

To address whether the MT induced fragmentation correlates with impairment of the energetic capacity of MT, we measured the mitochondrial oxygen consumption rate (OCR) and the MT transmembrane potential following treatment with Mito-C (FigS6). No significant changes in MT respiration or membrane potential are observed in treatment between 15 minutes and 2h (FigS6a, S6b and S6c). Furthermore, no effect on MT mass was observed (FigS6d and S6e). These data reveal that the observed phenotypes are due to changes in mitochondria morphodynamics and suggest that the morphology and function of the MT network are not necessarily coupled. To determine the contribution of NEET proteins to the observed effects of Mito-C, we analyzed the impact of siRNA-mediated knock down of each NEET protein transcript (Fig2a, 2b and 2c) on MT morphology (Fig2d). Reducing NAF-1 protein expression causes a significant increase in MT network symmetry, as measured by MT skewness while reducing MitoNEET or MiNT protein levels do not cause significant changes (Fig2e). To better assess the role of NAF-1 protein in MT morphodynamics we analyzed the MT network in cells stably expressing shRNA designed to reduce NAF-1 expression (Fig2f, g and h)). Electron microscopy analysis show an increase of MT fragmentation when NAF-1 protein is reduced, and this effect is rescued upon restoration of NAF-1 protein expression (Fig2f and 2h).

During mitochondrial fission, the dynamin related protein DRP1 is recruited to ER-MTcs to trigger the fission process [4,18], but the mechanisms that result in its enrichment and/or stability at the ER-MTcs remain poorly understood. Interestingly, NAF-1 colocalizes partially with DRP1 at ER-MTcs (Fig3a) and fluorescence image analysis shows that 15 minutes of Mito-C treatment leads to increased recruitment of DRP1 at mitochondrial surface (Fig 3b and 3c). This observation is confirmed by subcellular fractionation experiments (Fig 3d and 3e). Time course analysis of DRP1 expression following Mito-C treatment also showed an increase in total DRP1 protein (FigS7a and S7b), without any observed changes in phosphorylation at Serine 616 (DRP1<sup>S616</sup>), (FigS7a and S7c) a post-translational modification known to promote mitochondrial fission during mitosis [21,22]. To assess whether the effects of Mito-C on mitochondrial morphology were directly connected to DRP1 mitochondrial recruitment and function, we co-expressed a dominant negative version of DRP1 (*drp1K38A*) and mCherry, to discriminate between transfected and non-transfected cells. Expression of

*drp1K38A* is known to inhibit mitochondria fission [4,23]. We observed that Mito-C treatment causes the expected fragmented MT phenotype in non-transfected cells (NT, Fig 3f), while this effect was inhibited by expression of *drp1K38A*, which rather resulted in a hyperfused mitochondrial network both in presence and absence of Mito-C (Fig 3f and 3g). These data demonstrate that Mito-C and NAF-1 regulate MT fission in a manner dependent on DRP1 recruitment at MT, presumably at ER-MT contact sites.

Further investigation of the Mito-C stimulated MT fragmentation phenotype examined the level of isoforms of the MT-associated membrane dynamics regulator OPA1 (optic atrophy 1 protein). Long forms of OPA1 (L-OPA1) are associated with fusion [24,25] while shorter forms (S-OPA1) are considered fission mediators [26]. Interestingly, Mito-C treatment induces a time dependent decrease in L-OPA1 and a concomitant accumulation of S-OPA1 further confirming Mito-C and thereby NAF-1 function shifts the mitochondrial dynamic equilibrium toward a fission state (Fig S7c and S7d).

To go further, we investigated the possible role for SAMM50, a protein which localizes to the mitochondrial outer membrane and at ER-MTcs [27] and has been reported to interact with DRP1, promote its recruitment at MT surface and induce DRP1-dependent mitochondrial fission through an unknown mechanism (Ott *et al.*, 2012; Jian *et al.*, 2018, Liu *et al.*, 2016; Jian *et al.*, 2018). Interestingly, we show that siRNA downregulation of SAMM50 (FigS8a and S8b) increases DRP1 protein levels (FigS8a and S8c) and results in fragmentation of mitochondrial network (FigS8d and S8e). These phenotypes are strikingly similar to those we observed for the knock down of NAF-1 (Fig2 and S7). Taken together, these data suggest that NAF1 is essential for DRP1 dependent MT-fission via regulation of the mobilization of DRP1 at the ER and mitochondria interface.

Several viruses are known to modulate mitochondrial morphogenesis to result in elongation as a necessary part of their replication cycle [30]. These include Dengue virus, HIV-1, Sendai virus and SARS coronavirus which have been shown to promote elongation of the MT network in infected cells [31–34]. The effects of Mito-C and reduced NAF-1 protein expression on MT morphodynamics motivated an investigation into the possible effect of Mito-C upon the replication of specific viruses. Interestingly, we show that Mito-C treatment significantly reduces Dengue viral titer by more than one log (Fig4a) and concomitantly inhibits Dengue virus induced MT hyperfusion (Fig4b), We obtained similar results with Mito-C treatment of cells infected with two related flaviviruses Zika and West Nile virus (Fig4c and d). Our results suggest that Mito-C treatment and inhibition of NAF-1 function, through effects of MT fission counteract replication of viruses reliant upon elongated MT morphogenesis for successful replication. Conversely, hepatitis B virus (HBV) is described to induce



mitochondrial fragmentation [35]. Interestingly, treatment with Mito-C failed to impair HBV replication cycle, as indicated by the quantification of the surface antigen (HBsAg) and relaxed circular DNA (rcDNA) in the supernatant from infected cells (FigS9) thus providing a clue on the specificity of Mito-C anti-viral properties, based on mitochondrial morphodynamics phenotypes. Altogether, our data suggest that Mito-C is an anti-viral compound specific against viruses that enhance mitochondrial fusion as a necessary part of their replication cycle.

## Discussion

Here we report a novel small molecule (Mito-C) that targets the [2Fe-2S] lability/cluster transfer function of the three NEET proteins. Mito-C stimulates rapid and reversible mitochondrial fragmentation and the importance of the NEET protein NAF-1 for this effect is demonstrated by reduction of its expression recapitulating the fragmented mitochondrial phenotype. NAF-1 colocalizes with DRP1 at the ER-MTcs and the effect of Mito-C is dependent upon DRP-1 recruitment to the surface of mitochondria. Mito-C also stimulates MT fragmentation concomitant with accumulation of S-OPA1 a cleavage product of the fusion mediator OPA1 known to mediate MT fission. The therapeutic relevance of these observations is illustrated by the effect of Mito-C against replication of viruses reliant upon altered MT morphogenesis for successful replication.

Our present data suggest that beside intracellular iron levels modulation, NAF-1 protein can participate in the regulation of DRP1 recruitment to ER/ MT interface. If and how NAF-1 [2Fe-2S] cluster lability/ transfer at the ER-MT contact sites regulates mitochondrial morphogenesis remains to be elucidated. However, it is interesting to note that NEET proteins have been associated to different pathological conditions in which MT morphology as well as ER-MT contact site alterations appear to be a frequent signature: metabolic disorders, neurological, cancer [36–39] and now infections.

Interestingly enough, NAF-1 has also been shown to physically interact with Beclin1, a key protein of the class III PI3Kinase required for autophagosome biogenesis [40]. Autophagosomes have been proposed to form at the ER-MTcs [41] and several pathological conditions, including infections activate the autophagic pathway [33,34]. Here we highlight a function of NAF-1 at the sites of MT fission machinery recruitment suggesting that NAF-1 may participate in different processes reliant upon ER-MTcs machinery.

A role for mitochondrial morphodynamics, as well as MT-ER interface regulation, in viral replication and/or host response is highlighted here by the actions of Mito-C upon Dengue virus replication, while the lack of efficacy against HBV doesn't preclude the activity against a diverse array of viruses, and the observed inability of the virus to stimulate mitochondrial network elongation in treated cells. Eliciting an anti-viral effect by targeting human NEET proteins opens important new perspectives in antiviral research.

Our study points to the ER-MTcs as regions of intracellular communication integrating stress conditions such as viral infection and highlights NAF-1 as an important therapeutic target in diseases where altered mitochondria dynamics is implicated in disease etiology. The novel chemical class to which Mito-C belongs may prove a powerful tool to modulate mitochondrial dynamics in such pathological situations.

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**Author contributions:**

D.M., I.P.C., B.d.C., and E.Mo. conceived the project. D.M. designed, carried out the bulk of biochemical and cell biology experiments, organized the bulk of data, made statistical analyses and wrote the manuscript. I.P.C. contributes to some of the biochemical and cell biology experiments, analyzed immunofluorescence experiments on infected cells, participated in electron microscopy experiments and participated in manuscript preparation. H.B.M. performed electron microscopy and iron-sulfur clusters release experiments. L.R. performed some of the contact sites related experiments. O.K. and Y,-S, S generated and characterized stable shCisd2/NAF-1 cell lines. A.H. helped out for cytometry experiments and analyses. L.L. performed iron-sulfur clusters release experiments. P.R. and S.J. performed HBV experiments. A.R. performed Dengue, Zika and West Nile viruses infection experiments and edited the manuscript. J.V. designed part of the research. P.C., F.G., E.Me., P.M. and R.N. designed part of the research and wrote the manuscript. B.d.C and E.Mo. co-supervised the study, designed research and wrote the manuscript.

## Figures Legends

### Figure 1: Mito-C, a new chemical compound targeting NEETs proteins, induces mitochondrial network fragmentation

**a**, The structure of Mito-C **b**, HeLa cells were treated with DMSO or 2 $\mu$ M Mito-C for 15 min and immunostained with anti-TOMM20 (green) antibody and DAPI; cropped areas show the mitochondria symmetry changes. **c**, quantification of skewness of the TOMM20 signal from single cells (n=100). **d**, profile of [2Fe–2S] cluster release from purified recombinant NAF-1 was determined in untreated control or in presence of Mito-C and its inactive closely related analogue MITO-N by monitoring absorbance at 458 nm as a function of time. **e**, time lapse video-microscopy on HeLa cells transfected with NAF-1-mRFP (red) and treated with <sup>fluo</sup>Mito-C (green). The distance/intensity fluorescence quantification graph illustrates the codistribution of <sup>fluo</sup>Mito-C and NAF-1-mRFP. All scale bars = 10 $\mu$ m. For evaluating significance of differences observed in c, t-test was used (\*\* indicates p<0.0001); for differences observed in e one-way Anova followed by Dunn's post-test was used (\*\* indicates p<0.0001).

### Figure 2: Knocking down NEETs causes mitochondrial fragmentation

**a**, western blot analysis of MitoNEET expression in HeLa cells transfected with *CISD1* siRNA; Bar Chart (right panel) shows replicate quantifications (n=3) of MitoNEET expression. **b**, western blot analysis of NAF-1 expression in HeLa cells transfected with *CISD2* siRNA ; graph (right panel) shows the replicates quantifications (n=3) of NAF-1 expression. **c**, q-PCR analysis of *CISD3* mRNA levels from HeLa cells transfected with siRNA targeting *CISD3* (n=9). **d**, HeLa cells transfected with siRNA to reduce expression of MitoNEET (*CISD1*), NAF-1 (*CISD2*) or MiNT (*CISD3*) and immunostained with anti TOMM20 antibody. **e**, Single cell skewness quantification from images as shown in d, (n =125-130 cells). **f**, Electron microscopy pictures from 150 cells in transfected as described in g. **g**, Western blot analysis of *NAF-1* expression in cells stably transfected with control shRNA, shRNA targeting reduction of NAF-1 protein expression, or shRNA targeting reduction of NAF-1 protein expression complemented with plasmid derived expression of NAF-1; western blot quantification of NAF-1 is shown on the accompanying bar chart (n=3). **h**, Quantification of mitochondrial length in EM images from cells transfected as described in g. (n=260-270)). To evaluate significant differences observed in a, b, c, and g a t-test was used (for a \*\* indicates p<0.001; for b \* p is 0.045; c \*\* p is 0.005); for differences observed in e and f one way

Anova followed by Bonferroni's post-test was used (\*\* indicates  $p < 0.0001$ ); in i one way Anova followed by Dunn's post-test was used (\*\* indicates  $p < 0.0001$ ). Immunofluorescence scale bars = 10  $\mu$ m. Scale bars from EM analyses = 0.5  $\mu$ m.

### Figure 3: Mito-C causes DRP1 dependent mitochondrial fission

**a**, HeLa cells were transfected with NAF-1-GFP plasmid (green) and immunostained with antibodies to PTIP51 (ER-MT contact site marker, blue) and DRP1 (red); three color merged image is shown in the far-right panel with arrowheads indicating the white triple colocalization domains. **b**, HeLa cells treated or not, with 2  $\mu$ M Mito-C for 15 min where immunostained with anti-TOMM20 (red) and anti-DRP1 (green); arrowheads in the far-right panel indicate recruitment of DRP1 onto the mitochondrial surface (TOMM20). **c**, Quantification of DRP1 signal on TOMM20 positive structures (n=45). **d**, Western blot analysis of DRP1 and TOMM20 protein in cytosolic and mitochondrial fractions treated or not, with 2  $\mu$ M Mito-C as indicated. **e**, Quantification of western blots showed in d and expressed as a distribution of DRP1 in the cytosolic and mitochondrial fractions (n=5). **f**, HeLa cells were transfected with DRP1K38A mutant and mCherry and treated or not, with Mito-C (T for transfected, NT for not transfected). **g**, quantification of the mitochondrial phenotypes observed in f (30-35 images each with an average of 15-20 cells from triplicate independent experiments were analyzed). To evaluate significant differences observed in c, Mann Whitney was used (\*\* p is 0.003); To evaluate statistical differences shown in g, a one-way Anova test was used (\*\* indicates  $p < 0.0001$ ). All scale bars = 10  $\mu$ m.

### Figure 4 Mito-C counteracts flaviviruses replications

**a, c, d**, HuH7 cells were treated with Mito-C at the indicated concentrations and infected with Dengue (a), West Nile (c) and Zika (d) viruses. Infectious titers are presented as % of control in Mito-C concentration of 2 and 10  $\mu$ M conditions, upon 72 h of viral infection (n=3). **b**, HuH7 cells were treated with Mito-C, infected with Dengue virus and fixed after 72h of infection. Cells were then immunostained for endogenous TOMM20 (green channel) and viral Dengue NS5 protein (Red). Cropped areas illustrate the mitochondrial morphology in described conditions. To evaluate significance of differences observed in a, c and d, two-way Anova followed by Bonferroni's post-test was used. NS, for non-significant, in a \*\* indicate  $p = 0.006$  and \*\*\*  $p = 0.0003$ , in c \*\* for  $p = 0.009$ , in d \* for  $p = 0.025$ . Scale bars = 10  $\mu$ m.

### **Figure S1 Signal skewness as a measure of mitochondrial symmetry**

**a**, schematic view of spatial pixels distribution and skewness quantification: the higher distribution symmetry corresponds to the higher skewness value. **b**, HeLa cells were treated with increasing concentrations of Mito-C for 15 minutes, fixed and immunostained for TOMM20 and analyzed by light microscopy to evaluate skewness. **c**, quantification of signal skewness informs acquisitions illustrated in **b**, (n=45-50). To evaluate significance of differences observed in **c**, one-way Anova followed by Bonferroni's post-test was used (NS for non-significant, \*\*\* for  $p < 0.0001$ ). Scale bar = 10  $\mu\text{m}$ .

### **Figure S2: Mito-C treatment is reversible and does not induce cell death**

**a, b**, HeLa cells treated for 24h with increasing concentrations of Mito-C were stained with propidium iodide (PI) and annexin V and analyzed by cytometry. **c**, HeLa cells were treated with DMSO or 2  $\mu\text{M}$  Mito-C for 15min, with 2  $\mu\text{M}$  Mito-C for 15min than washed-out overnight, treatments were blocked by fixation and mitochondria visualized by immunostaining with anti-TOMM20. **d**, quantification of mitochondrial skewness following treatment or treatment followed by removal of compound as illustrated in **c** (n=120-140, where n are cells). To evaluate significance of differences in **b** we used t-test, NS for non-significant. In **d** one-way Anova was used (NS for non-significant, \*\* indicates  $p < 0.001$  and \*\*\* $p < 0.0001$ ). Scale bar = 10  $\mu\text{m}$ .

### **Figure S3: Mito-C localizes to both mitochondria and Endoplasmic Reticulum**

**a**, HeLa cells were treated with <sup>fluo</sup>Mito-C (green), incubated with Mitotracker Redox (red) and analyzed by live microscopy. **b**, HeLa cells were transfected with Sec61 $\beta$ RFP (as ER marker), treated with <sup>fluo</sup>Mito-C (green), and analyzed by live microscopy Scale bar are 10  $\mu\text{m}$ .

### **Figure S4: Mito-C impacts Fe-S clusters release from MitoNEET and MiNT proteins**

**a** and **b**, profile of [2Fe–2S] clusters release from purified recombinant MitoNEET and MiNT were determined in presence or absence of Mito-C and Mito-N (an inactive form of Mito-C) via monitoring their absorbance at 458 nm as a function of time. For differences observed in both **a** and **b** one-way Anova followed by Dunn's post-test was used (\*\*\* indicates  $p < 0.0001$ ).

#### **Figure S5: NAF-1 localizes to the ER-MT contact sites**

**a**, HeLa cells were transfected with Sec61 $\beta$ RFP (ER marker, red) and NAF-1-GFP (green), fixed and analyzed by confocal microscopy. **b**, HeLa cells were transfected with NAF-1-GFP (green), fixed, immunostained with TOMM20 antibody (red) and analyzed by confocal microscopy. **c**, HeLa cells were transfected with Sec61 $\beta$ RFP (ER marker, red), and NAF-1GFP (green), fixed, immunostained for PTIP51 (ER-MT contact sites marker, blue) and analyzed by confocal microscopy. Scale bars = 10  $\mu$ m.

#### **Figure S6: Mitochondrial mass, membrane potential and oxygen consumption rate of Mito-C treated cells**

**a**, oxygen consumption rate (OCR) of cells treated with Mito-C 2, 10 and 20  $\mu$ M were measured by Seahorse® technique. Measurements start before starting the treatment, arrow indicates the Mito-C injection. **b**, to evaluate the mitochondrial potential, cells were treated with Mito-C for 24hrs at the indicated range of concentrations and stained with Mitotracker Redox and analyzed by cytometry. **c**, to evaluate the mitochondrial potential, cells were treated with Mito-C at 2 $\mu$ M over a time course and then stained with Mitotracker Redox and analyzed by cytometry. **d**, To evaluate the total mitochondrial mass over a time course of treatment, HeLa cells were treated with DMSO or Mito-C at 2 $\mu$ M for the time duration indicated, stained with Mitotracker green and analyzed by cytometry. **e**, To evaluate the total mitochondrial mass at a fixed time point following treatment with an increasing range of concentrations of Mito-C cells were treated with DMSO or Mito-C for 24hrs at concentration indicated, stained with Mitotracker green and analyzed by cytometry

#### **Figure S7: Expression of DRP1 and OPA1 during Mito-C treatment**

**a**, Western blot analysis of DRP1 and phospho<sup>616</sup>DRP1 expression in total extracts from cells treated with 2 $\mu$ M Mito-C for the times indicated. **b**, **c**, Quantifications by Western blot with anti-DRP1 and anti-phospho<sup>616</sup>DRP1 (n=5). **d**, Western blot analysis of OPA1 expression in total extracts from cells treated with 2 $\mu$ M Mito-C during time course indicated; long and short isoforms are indicated. **e**, **f**, Quantification of Western blot for long and short isoforms of OPA1 shown in d. To evaluate significance of differences observed in b,c,e and f we used a one-way Anova followed by Dunn's post-test in a and Bonferroni's post-test in c, e and f (ns for non-significant \*p<0.01, (\*\*p<0.001\*\*\*p<0.0001).



### **Figure S8: Knock down of SAMM50 induces mitochondria fragmentation**

**a**, Western blot analysis of SAMM50 and DRP1 protein in total extracts from cells transfected with siRNA for SAMM50 ER-MT associated protein (siSAMM50) or with control siRNA (siCTRL). **b** and **c**, Western blot quantification for SAMM50 and DRP1 as shown in **a** (n=5). **d**, HeLa cells were transfected with siRNA for SAMM50 ER-MT associated protein (siSAMM50) or with control siRNA (siCTRL), fixed and immunostained with anti-SAMM50 (red) and mitotracker (green). Scale bar = 10µm. **e**, quantification of mitochondrial phenotype. For each image the % of cells displaying a fragmented or filamentous (indicated as normal) phenotype is shown. Data were generated from analysis 30 images containing 15-30 cells each. To evaluate significance of differences observed in **b** and **c** we used an unpaired t-test, in **b** \*\* p is 0.001, in **c** \*\* p is 0.008. In **e** one-way Anova followed by Dunn's post-test was used (\*\*p<0.0001).

### **Figure S9: absence of MITO-C effect on Hepatitis B virus replication**

**a**, Relative secretion of HBsAg (from Hepatitis B virus (HBV)) in dHepaRG cells treated post-infection with 10 µM of Mito-C or the FXR agonist GW4064. **b**, Relative secretion of HBV viral DNA in dHepaRG cells treated post-infection with 10 µM of Mito-C or GW4064 and quantified by quantitative PCR.

## Materials and Methods

### Cell culture, transfection and treatment with molecules

HeLa cells (ATCC) were grown in Minimum Essential Medium (MEM, Gibco), supplemented with GlutaMAX and 10% FCS at 37°C and 5% CO<sub>2</sub>. Huh7 cell were maintained in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1x non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin (all from GIBCO, Life Technologies) and 10% fetal calf serum (Capricorn). INS-1E-cells were grown in RPMI 1640 with 11.1 mmol/L D-glucose supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 50 mol/L mercapto-ethanol. INS-1E-knockdown cell lines were generated as described previously [42] and maintained in the same medium as control cells with Puromycin (1g/mL). Human HepaRG cells were grown in William's E Medium supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 5 µg/mL insulin, 25 µg/mL hydrocortisone hemisuccinate, and 10% FCS at 37°C and 5% CO<sub>2</sub>. Plasmid transfection in HeLa cells were performed using FuGENE® HD Transfection Reagent (Promega, E2311) following the manufacturer's instructions. Analyses were performed 24 h after transfection. Constructs used include NAF-1-GFP and NAF-1-RFP which were generated by cloning the ORF sequence NM\_001008388.4(CISD2) into pcDNA3.1(+)-C-eGFP and pmCherry-N1 Vector by GeneScript; the dominant mutant K38A DRP1 was a gift from D. Arnoult (INSERM UMR-S 1014, Hôpital Paul Brousse, Villejuif, France); Sec61β-RFP was a kind gift from T. Rapoport (Harvard University, Cambridge, MA, USA). siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen, 12323563) following the manufacturer's guidelines. Analyses were performed 72 h after transfection. siRNAs used were: siCTRL (Qiagen, AllStars Negative Control siRNA SI03650318); siCISD1 (Qiagen, SI04758194); siCISD2 (Qiagen, si04985855); siCISD3 ( Qiagen, SI04758187); SAMM50 (Dharmacon, mix 1:1 J-017871-18 and J-017871-19); Non-targeting (Dharmacon, D-001810-10). Cells were treated with Mito-C, Mito-N and <sup>fluor</sup>Mito-C compounds at the indicated concentrations diluted directly in culture media, as negative control the same amount of DMSO was used.

### Protein extracts, membrane fractionation and Western blot analyses

For total protein extracts, cells were washed twice with 1XPBS and then directly lysed on ice with 1X Laemmli buffer (60mM Tris-HCl pH=6.8, 2% SDS, 10% Glycerol, bromophenol blue, supplemented with fresh added 100mM DTT final), lysates were incubated for 10 min at

90°C. For mitochondrial and cytosolic fraction preparations, after treatment HeLa cells were washed twice and gently scraped in cold PBS. Cell pellets were recovered via centrifugation at 600g for 5 min. Cells were gently re-suspended in M buffer (440 mM mannitol, 140mM sucrose, 40mM HEPES, 1mM EDTA, 2mg/ml fatty acid free BSA and protease/phosphatase inhibitor cocktail) and placed on ice for 10 minutes. After homogenization with Dounce homogenizer (20 stroke), the lysate was centrifuged at 600g for 5min to pellet nuclei and recover the post nuclear supernatant (PNS). The PNS was then centrifuged at 7200 g for 15 min at 4°C to collect the mitochondrial enriched fraction and the supernatant (cytosolic fraction). For routine SDS-PAGE, precast gradient gels (4-20% Tris-Glycine, Invitrogen) were used and home-made 7.5% Tris-Glycine gels for OPA-1 detection. Separated proteins were transferred onto PVDF membranes. Membranes were blocked with BSA 3%/ TBS/0.1% tween for 1h and incubated with primary antibodies overnight at 4° in 2% BSA/TBS/0.1%tween. Immunoblot analysis was performed by chemiluminescence (Millipore) in a ChemiDoc MP Imaging System (Bio-Rad). Quantification of band intensities were carried using Image J software.

### **Immunofluorescence and confocal microscopy**

For immunofluorescence analysis, cells were plated on 12-mm glass coverslips and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After 3 washes of 20 min each in PBS, cells were blocked with fetal calf serum (10%) in PBS for 30 min. Incubation with primary antibodies was performed in permeabilization buffer (0.05% saponin in blocking buffer). Coverslips were mounted on microscope slides using home-made Mowiol mounting medium with or without DAPI. Images were obtained using a 63x oil-immersion objective with Leica TCS SP5 confocal microscope, using a 405 nm diode laser line exciting DAPI, a 488 nm argon laser line exciting Alexa Fluor 488, a 561 nm diode laser line for Alexa Fluor 546 and laser He/Ne 633 nm for Alexa 647. Acquisitions were done in sequential mode and fluorescence acquired in separated channels. For some experiments, optical sections were acquired with a 63x/1.4 Oil immersion objective using the LAS-X software and fluorescent pictures were collected with a PMTs GaAsP hybride camera (Hamamatsu).

### **Image analysis and statistics of data**

Image analysis was performed using Image J software. For mounting representative images, background was reduced using brightness and contrast adjustments applied to the whole image. For obtaining skewness measurements a circle of 6µm diameter was drawn and moved near the nucleus of each cell, then skewness values were obtained from the set

measurement plugin option “skewness” of ImageJ software. For analyzing the recruitment of DRP1 on mitochondria, a mask on the fluorescence in TOMM20 channel was used to define the region to measure the total intensity of the DRP1 signal in the DRP1 channel. For statistics of data we used GraphPad Prism software. After evaluation of mean, standard deviation and standard errors we evaluate data distributions using normality test: KS normality test, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test. Data were processed as normally distributed when at least 2 out of 3 test resulted positive, otherwise they were processed as non-parametric distributions. Gaussian distributions were analyzed with the more appropriate t-test or Anova and non-Gaussian distributed sets of data were evaluated with the more appropriate non parametric test, Mann Whitney or ANOVA, as specified in figure legends.

### **Antibodies used and dilutions**

The primary antibodies used are the following: mouse anti-TOMM20 (BD Biosciences 612278, 1:1000 for WB, 1:400 for immunofluorescence); rabbit anti-MitoNEET (Proteintech 16006-1-AP, 1:3000); rabbit anti – NAF-1 (Proteintech 13318-1-AP, 1:3000); mouse anti-DRP1 (BD Biosciences 611112, 1:1000); rabbit anti- DRP1 P616 (Cell signaling 3455, 1:1000); rabbit anti-PTPIP51 (Novus biological NBP1-84738, 1:1000 for WB, 1:200 for immunofluorescence); mouse anti- OPA-1 (BD Biosciences 612607, 1:1000); rabbit anti SAMM50 (Sigma HPA034537, 1:1000 for WB, 1:200 for immunofluorescence); mouse anti beta-actin (Abcam ab8226, 1:1000); rabbit anti-Dengue virus NS5 protein (GeneTex GTX124253, 1:1000). For secondary antibodies, we used for immunofluorescence an Alexa 488 conjugated donkey anti-mouse (Invitrogen A21202, 1:800), Alexa 488 conjugated donkey anti-rabbit (Invitrogen A21206, 1:800), Alexa 647 conjugated donkey anti-mouse (Invitrogen A31571, 1:800), Alexa 647 conjugated donkey anti-rabbit (Invitrogen A31573, 1:800). For western blotting we used Secondary HRP conjugate anti-rabbit IgG (GE Healthcare, 1:10000) and HRP conjugate anti-mouse IgG (Bio-Rad 1:10000).

### **RNA extraction and quantification**

RNA was extracted from cells using NucleoSpin RNA kit (Macherey-Nagel, 740984.50) according to the manufacturer's instructions. cDNA was made from 1 µg of RNA using M-MLV reverse transcriptase (Life Technologies, 28025013). Detection of MiNT transcript was performed by qPCR with iTAQ universal SYBR Green supermix (Applied biosystem) using

the following primers for 5'-GCAGGGAAAACCTACAGGTG – 3' and 5'-TGAGTGGAGATAGGCCAGTG – 3' (Eurofins) in a qTOWER machine (Analytik Jena).

### **Flow cytometry analyses**

Cells were treated as indicated in each figure legend. After treatment, cell death was quantified using Annexin V-FITC/Propidium Iodide (PI) assay according to the manufacturer's protocol (Annexin V-FITC Apoptosis Detection Kit II, 556570, BD Pharmingen™). For mitochondria analysis we used MitoTracker Red CMXRos (Thermo Fisher, M7512) and MitoTracker Green FM (Thermo Fisher, M7514). Data were analyzed by a LSRFortessa™ flow cytometer (BD Biosciences, San Jose, CA) and processed using Cell Quest software (BD Biosciences) and FlowJo software (FLOWJO, LLC).

### **Oxygen consumption rate and mitochondrial activity measurements**

For measurements of mitochondria activity, HeLa cells were seeded at a density of 6000/well in a XF96 cell culture microplate. 16 hours later cells were balanced for 1h in un-buffered XF assay media (Agilent Technologies) supplemented with 2 mM Glutamine, 10 mM Glucose and 1 mM Sodium Pyruvate, then oxygen consumption rate were measured by Seahorse bio analyzer. Values were taken every 3 minutes and Mito-C was injected during the assay at reading time of 10 min (indicated by the arrow in the figure S6a) at the final concentrations of 2, 10 or 20  $\mu$ M. The data were normalized to protein content measured in each well using BCA assay (Thermo Fisher Scientific) according to manufacturer's instructions

### **Mito-C synthesis**

Mito-C (2-[(3,4-Dimethoxybenzoyl)amino]-6,6-dimethyl-5,7-dihydro-4H-benzothiophene-3-carboxylic acid) was synthesized by Charles River company as following: a reaction vessel was charged 4,4-dimethylcyclohexanone (CAS: 4255-62-3, 5.00 g, 39.6 mmol), methyl cyanoacetate (CAS: 105-34-0, 3.8 ml, 43.6 mmol), diethylamine (CAS: 109-89-7, 2.0 ml, 19.8 mmol) and sulfur (CAS: 7704-34-9, 1.52 g, 47.5 mmol). The reaction was solvated in methanol (25 ml) and set to stir at RT. The reaction mixture was stirred at room temperature for 60 hours. The volatiles were removed under reduced pressure and the residue was purified by flash chromatography on silica gel (eluting with 0-20% EtOAc in isohexane) which afforded *methyl 2-amino-6,6-dimethyl-4,5,6,7-tetrahydrobenzo[b] thiophene-3-carboxylate* as a pale yellow solid (7.22 g, yield 76%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz):  $\delta$  = 5.92

(s, 2H), 3.79 (s, 3H), 2.69 (t, J=6.4 Hz, 2H), 2.27 (s, 2H), 1.48 (t, J=6.4 Hz, 2H), 0.98 (s, 6H). To a solution of methyl 2-amino-6,6-dimethyl-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (Preparation #1, 200 mg, 0.84 mmol) in DCM (5.0 ml) was added DIPEA (CAS: 7087-68-5, 220  $\mu$ l, 1.25 mmol) and 3,4-dimethoxybenzoyl chloride (CAS: 3535-37-3, 120  $\mu$ l, 1.00 mmol). The reaction mixture was stirred at RT overnight. The resulting mixture was diluted with DCM and water. The two phases were separated. The organic layer was passed through a phase separator and the solvent was removed under reduced pressure. The residue was dissolved in THF (4.0 ml) and MeOH (2.0 ml). To the solution was added LiOH aq. (CAS: 1310-66-3, 2.0M, 1.7 ml, 3.36 mmol). The reaction mixture was stirred at 50 °C for 2 hours. The mixture was allowed to cool to RT and acidified with 1N aqueous HCl solution. The reaction mixture was then extracted with EtOAc. The organic phase was washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure. Purification by RP-HPLC. RP-HPLC purification condition: Column XSELECT CSH Prep C18 19x250mm, 5 $\mu$ m. Mobile phase: MeCN in water (0.1% HCOOH), Flow rate: 20 ml/min; Wavelength: 210-260 nm DAD. Sample injected in DMSO (+ optional formic acid and water), 22 min non-linear gradient from 10% to 95% MeCN, centered around a specific focused gradient. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400MHz):  $\delta$  = 13.34 (br s, 1H), 12.36 (s, 1H), 7.49 - 7.45 (m, 2H), 7.18 (d, J=8.4 Hz, 1H), 3.86 (s, 3H), 3.85 (s, 3H), 2.75 (t, J=5.9 Hz, 2H), 2.42 (s, 2H), 1.50 (t, J=6.3 Hz, 2H), 0.98 (s, 6H). LC/MS (Table 1, Method A) R<sub>t</sub> = 5.31 min; MS *m/z* 390 [M+H]<sup>+</sup>. Compounds were > 95% pure, reconstituted in 100% DMSO (Sigma) and diluted in cell culture medium for assays.

### **Flavivirus virus infection and estimation of virus titers upon Mito-C treatment**

Dengue virus serotype 2 strain New Guinea C was produced as previously described and infectious titers determined by limiting dilution assay using Huh7 cells [43]. Zika virus strain MR766 was obtained from the European Virus Archive (EVAg, France). West Nile virus strain New-York99 was a kind gift of J. Schmidt-Chanasit (Hamburg, Germany). Zika and West Nile viruses were passaged once on C6/36 cells and stocks were prepared by virus amplification in VeroE6 cells. Virus stock titers were determined by plaque assay. In brief, VeroE6 cells were infected with serial dilutions of virus supernatants. Two hours post-infection inoculum was replaced by serum-free MEM medium (Gibco, Life Technologies) containing 1.5% carboxymethyl cellulose (Sigma Aldrich). At different days post infection (day 3 for West Nile virus, day 4 for Zika virus, day 8 for Dengue virus) cells were fixed by addition of formaldehyde to a final concentration of 5%. Cells were stained with crystal violet solution (1% crystal violet, 10% ethanol in H<sub>2</sub>O) for 30 min at room temperature and rinsed extensively with H<sub>2</sub>O. Infectious titers were calculated considering the corresponding dilution

factor. Huh7 cells ( $1 \times 10^5$ ) were infected with Dengue, Zika and West Nile viruses at a multiplicity of infection (MOI) of 0.1 TCID<sub>50</sub> or pfu per cell and simultaneously treated with 20  $\mu$ M of mito-C for 48 h. Supernatants were harvested, filtered through 0.45  $\mu$ M membrane and titrated by limiting dilution assay or plaque assay as described above. For immunofluorescence assays, Huh7 cells ( $1 \times 10^5$ ) were infected with Dengue virus at a MOI of 0.5 TCID<sub>50</sub> per cell and simultaneously treated with 20  $\mu$ M of Mito-C for 72 h.

### **HBV infection and viral growth estimation under Mito-C or GW4064 treatment**

Hepatitis B virus (HBV) inoculum was prepared from stably transfected HepG2.2.15 cell line as previously described [35]. HepaRG cell inoculation was performed with 100 genome-equivalent per cell in culture medium that contained 4%v/vPEG8000 (Sigma-Aldrich) for 24 h at 37°C. At day 2 post-infection, cultures were treated for 10 days with the following compounds Mito-C or GW4064 at indicated concentrations. HBs antigen (HBsAg) secreted into cell supernatants were quantified on the miniVIDAS apparatus using the VIDAS Ultra tests (Biomérieux, Marcy l'Etoile, France). Viral HBV DNA was extracted from cell supernatant using the QUIAamp MinElute virus spin kit (Qiagen, Courtaboeuf, France) on a QIAcube apparatus (Qiagen). Extractions were carried out following the manufacturer's recommendations. Eluates were directly used for quantification of secreted viral DNA by quantitative PCR experiments using primers for rcHBV DNA: forward 5'-GGGGAGGAGATTAGGTTAAAGGTC-3', reverse 5'-CACAGCTTGGAGGCTTGAACAGTGG-3' and the QuantiFast SYBR green PCR kit (Qiagen) on a LightCycler 480 II (Roche Applied Science).

### **Transmission electron microscopy**

Cells were fixed for 2h at room temperature with 2% glutaraldehyde in 0.1 $\mu$ M sodium cacodylate buffer at pH of 7.4. Samples were then rinsed three times of 10 min each in 0.1 $\mu$ M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 $\mu$ M sodium cacodylate buffer for 30 min. Contrast was done in uranyl acetate 1% for 30 min, then the samples were dehydrated in a graded series of ethanol ending with 100% ethanol. Samples were then embedded in EPON. About 70 nm sections were prepared with a Leica Ultracut 7 ultramicrotome. The sections were observed using a Philips CM120 electron microscope operating at 120 kV. Images were captured using a GATAN Orius 200 camera.

### **Fe-S cluster transfer assay**

The stability of NEET protein binding for its [2Fe–2S] cluster was determined from monitoring their characteristic absorbance at 458 nm as a function of time with ~66  $\mu$ M NAF-1 protein in 20 mM Tris–HCl pH 5.5, 100 mM NaCl on a Synergy 2 microplate reader (BioTek Instruments). NEET proteins were expressed and purified as previously described [44].



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